Melanocortin Ligands: 30 Years of Structure–Activity Relationship (SAR) Studies

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Abstract: The challenge of peptide and peptidomimetic research is the development of methods and techniques to improve the biological properties of native peptides and to convert peptide ligands into non-peptide compounds. Improved biological properties of peptides includes enhancement of stability, potency, and receptor selectivity, for both *in vivo* and *in vitro* applications. The design of a ligand with specific activity and desired biological properties is a complex task, and, to accomplish this objective, knowledge about putative interactions between a ligand and the corresponding receptor will be valuable. This includes interactions for both the binding and signal transduction processes. Structure–activity relationship (SAR) studies involve systematic modification of a lead peptide and are designed to provide insight into potential interactions involved in the formation of the ligand–receptor complex. It is desirable to have knowledge about both favorable and unfavorable processes that may occur in putative ligand–receptor interactions that result in either receptor stimulation or inhibition. Herein, we discuss various SAR studies that have involved melanocortin peptides over three decades and the information these studies have provided to the melanocortin field. © 2004 Wiley Periodicals, Inc. Med Res Rev, 24, No. 3, 325–356, 2004

Key words: obesity; G-protein coupled receptors; melanocortin receptors; melanotropin; structure–activity relationship; peptidomimetic

1. INTRODUCTION

The melanocortin receptor system consists of five receptor isoforms (MC1R–MC5R) identified to date, and belong to the superfamily of G-protein coupled receptors (GPCRs).[1–8] Comprehensive reports of the melanocortin receptor system have been reviewed elsewhere.[9–11] When stimulated by agonist ligands, the melanocortin receptors activate the cyclic adenosine monophosphate (cAMP)

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Table I. Amino Acid Sequence of Endogenous Melanocortin Agonists

Peptide	Sequence
α-MSH	Ac–$S^1Y^2S^3M^4E^5$ $H^6F^7R^8W^9$ $G^{10}K^{11}P^{12}V^{13}$–NH_2
β-MSH	AEKKDEGPYRME $H^6F^7R^8W^9$ GSPPKD–OH
γ-MSH	YVMG $H^6F^7R^8W^9$ DRFG-OH
ACTH	SYSME $H^6F^7R^8W^9$ GKPVGKKRRPVKVYPNGAEDESAEAFPLEF-OH

signal transduction pathway.[12] Melanocortin receptor isoforms appear to have developed very early in vertebrate evolution, indicating that these receptors play a vital role in physiological functions.[13] The naturally occurring agonists of the melanocortin receptors are α-, β-, and γ-melanocyte-stimulating hormones (α-, β-, and γ-MSH) and adrenocorticotropin hormone (ACTH), which are derived from posttranslational modification of the proopiomelanocortin (*POMC*) gene transcript.[14] The endogenous agonists (Table I) for the melanocortin receptors all contain a conserved His-Phe-Arg-Trp sequence that has been attributed to melanocortin receptor selectivity and stimulation.[15–18] Interestingly, the melanocortin receptor system is unique among GPCRs in that it possesses both naturally occurring agonists and antagonists. The melanocortin antagonists, agouti and agouti-related protein (AGRP), are the only two endogenous antagonists of GPCRs identified to date.[19–25] Recently, two additional protein families consisting of attractin/mahoganoid,[26,27] and the syndecans[28] were shown to affect coat coloration and body weight in Agouti mice that have aberrant overexpression of the agouti protein (A^y mice).[29,30] It has been suggested that the attractin/mahoganoid and syndecan protein families participate in the regulation of the melanocortin pathway by interacting with the endogenous melanocortin receptor antagonists agouti and/or AGRP upstream of the melanocortin receptors.[31]

The MC1R is expressed in melanocytes and is involved in skin pigmentation, animal coat coloration, and melanocyte function.[1,2,32,33] Polymorphisms in the *MC1R* gene have been attributed to the red hair phenotype, melanoma and non-melanoma skin cancer.[34–38] Because of the association of the MC1R with melanoma, the MC1R may prove to be beneficial in the prevention and treatment of certain forms of skin cancer.[39,40] Melanocytes appear to have additional functions than simply the production of melanin.[38,41–49] They are able to secrete a wide range of signal molecules, including cytokines, POMC peptides, catecholamines, and nitric oxide (NO) in response to UV irradiation and other stimuli.[33] The targets for these melanocyte secretory molecules may serve as regulators of a variety of functions yet to be determined. Recently, sebocytes isolated from an immortalized human sebaceous cell line were found to express MC1R that modulated interleukin-8 secretion, Bohm et al.[50] suggest that MC1 receptors may act as a modulator of inflammatory responses in the pilosebaceous unit. Herpin et al.[51] have used a selective small molecule agonist for the MC1R to demonstrated the role of MC1R in modulation of inflammation. The MC2R, which only responds to stimulation by the ACTH endogenous melanocortin agonist, is expressed in the adrenal cortex and adipocytes and is involved in steriodogenesis.[52–55] Recently, RT-PCR has provided evidence that mRNA for the MC2R is expressed in normal and malignant human skin cells, although the function of the receptors has yet to be determined.[56] The MC3R is found in the brain, heart, placenta, and the gut, and in addition to activating the adenylate cyclase pathway, the MC3R may also activate the inositol phospholipid/calcium signal transduction pathway.[6,7,57] Recent studies have implicated the involvement of the MC3R in the complex pathways of energy homeostasis.[58,59] Deletion of the *MC3R* gene from the mouse genome resulted in mice with increased fat mass, reduced lean mass, and higher feed efficiency than wild-type littermates, despite being hypophagic.[58] A mutation in the MC3R has been identified that is associated with severe obesity, providing additional support that the MC3R is involved in

energy homeostasis.[60] The MC4R has been detected in the rat and human penis, the rat spinal cord, hypothalamus, brainstem, and pelvic ganglion (major autonomic relay center to the penis).[61–63] Several lines of evidence support a role for the MC4R in energy and weight homeostasis, although the regulatory pathways of the MC3 and MC4 receptors are believed to be distinct. Deletion of the MC4R resulted in a mouse that develops an adult onset obesity syndrome associated with hyperphagia and type-2 diabetes,[64] similar to the Agouti mouse[65] (which ectopically expresses the endogenous antagonist agouti[66]). Identification of mutations in the *MC4R* and *POMC* genes in obese humans provides further support of the involvement of the MC4R in obesity.[67–71] In addition to the participation of the MC4R in weight homeostasis, it appears to be involved in sexual behavior and function.[63] The MC5R has the widest tissue distribution of all the melanocortin receptors.[72] The MC5R is found in both central and peripheral exocrine glands and tissues, and is involved in thermoregulation and exocrine gland function.[73]

Because of the participation of the melanocortin receptor family in a vast array of physiological functions, and particularly the involvement of the MC3R and MC4R in energy and weight homeostasis, these receptors have been the center of a large amount of research by both academic and industrial laboratories. The melanocortin ligands, both endogenous and synthetic, have been lead compounds in many structure–activity relationship (SAR) studies. Structure–activity studies are designed to provide insight into the types of interactions that occur in the formation of the ligand–receptor complex. It is desirable to gain knowledge about both the favorable and unfavorable processes that occur in ligand–receptor interactions that ultimately result in receptor stimulation (or inhibition). An objective of structure–activity studies is to aid in the design of ligands, with specific function (i.e., agonist or antagonist), *a priori* for a given receptor or receptor system. In this review, various SAR studies that have involved modifications of both endogenous and synthetic melanocortin ligands are presented. The studies discussed herein exemplify the rational design processes of peptidomimetic research and reveal insight these types of studies have provided to the melanocortin field.

2. TRUNCATION STUDIES OF MELANOCORTIN LIGANDS

Once a peptide lead has been identified, it is important to know which of the amino acid residues contribute to molecular recognition and receptor stimulation. Studies have been undertaken to determine the minimal sequence required to illicit a pharmacological response for the melanocortin agonists α-MSH [amino acid numbering throughout this review refers to the corresponding position of the amino acid residue in the sequence of α-MSH (Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2)] and the highly-potent analog NDP-MSH (Ac-Ser^1-Tyr^2-Ser^3-*Nle*^{4}-Glu^5-His^6-*D*-*Phe*^{7}-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2).[15–17,74–76] These studies involved selective removal (truncation) of N- and/or C-terminal residues, followed by evaluation of the truncated analogs for binding and/or functional activity. Figure 1 illustrates the truncation process for NDP-MSH and the results of truncation at the MC4R. In the classical frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassays, peptide activity was monitored by quantifying the amount of skin darkening that occurs in response to exposure to the peptide.[77,78] The minimal sequence required for biological activity was determined to be Ac-His^6-Phe^7-Arg^8-Trp^9-NH_2 for α-MSH$^{15–17}$ and Ac-DPhe^7-Arg^8-Trp^9-NH_2 for NDP-MSH in the skin pigmentation assay.[17,75] The NDP-MSH truncation results have been supported in more recent studies that utilized the cloned mouse MC1, MC3–MC5,[18,79] and the cloned human MC4[80] receptors. It should be noted that although the minimal NDP-MSH sequence required for activity was determined to be Ac-DPhe-Arg-Trp-NH_2, addition of histidine significantly increased potency (>100-fold) at each of the four mouse melanocortin receptors receptors.[18] Truncation studies of α-MSH using the frog skin bioassay revealed that residues 4, 10, and 12 contribute to the potency of the peptide and that residues 1–3, 5, 11, and 13 negligibly

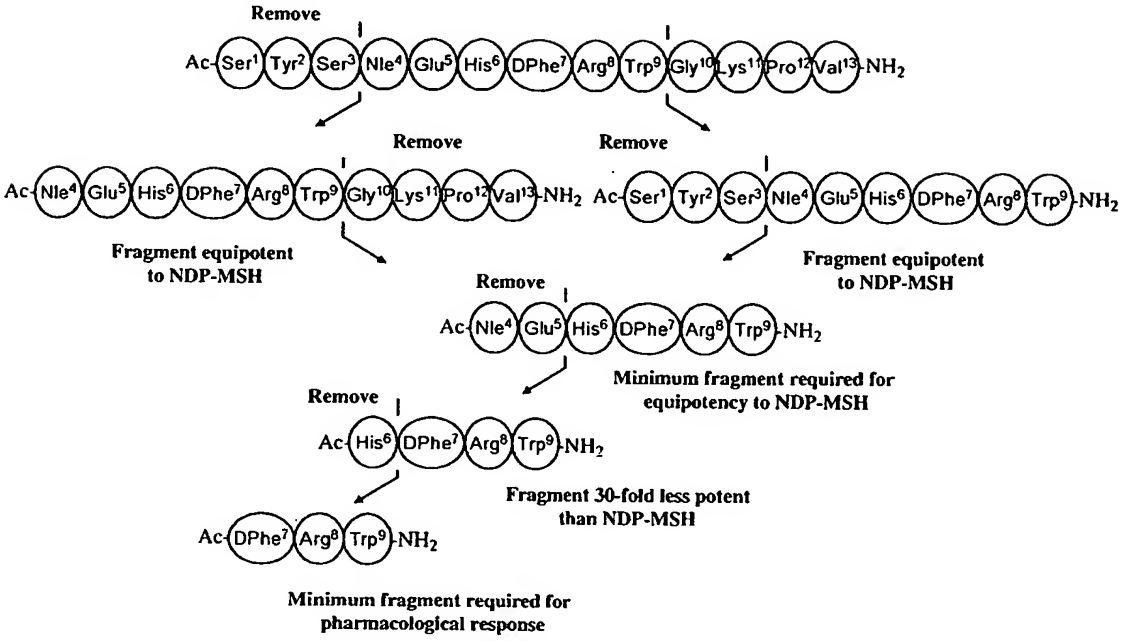


Figure 1. Primary structure of NDP-melanocyte-stimulating hormone (MSH) and truncated fragments. Amino acid residues are represented by individual spheres. Biological activity of NDP-MSH and truncated fragments is at the MC4R one.

effect potency.[15,81] Using the lizard skin bioassay, residues 1–3, 5, and 13 were identified not to be important for agonist potency.[16] These data suggest that residues 4–12 of MSH and residues 4–9 of NDP-MSH are the minimal residues required to retain potency equivalent to the lead peptides and the endogenous α-MSH agonist. Three predictable conclusions have been observed from these truncation studies regarding the importance of α-MSH and NDP-MSH amino acids: (1) the melanocortin peptides contain an essential core sequence that is required to elicit measurable biological activity; (2) the peptides contain important potentiating amino acids that are necessary to retain equipotency to the parent peptide; and (3) the peptides contain amino acids that contribute minimally to the potency of the ligands.

3. ALANINE SCAN STUDIES

Specific molecular interactions are postulated to occur between a ligand and its corresponding receptor for: (1) molecular recognition, (2) ligand binding, and (3) receptor stimulation to occur. It is important to determine which residues of the lead peptide are required for these ligand–receptor events to occur, especially if the scientific objective is to design a ligand with specific activity at a particular receptor system. A classical method used to determine the amino acid residues involved in these ligand–receptor events is the alanine scan method. Alanine scanning studies can complement truncation studies and aid in the identification of the residues responsible for, or contributing to, the biological properties of the native peptide important for molecular recognition and functional activity. There has been a complete alanine scan of α-MSH characterized using B16 murine melanoma cells (putative MC1R),[82] and more recently a complete alanine scan of γ-MSH characterized at the cloned human MC3–MC5 receptors.[83] The essential role that the melanocortin agonist core His-Phe-Arg-Trp sequence plays in peptide–receptor interactions was identified from both of these alanine scan studies. The results from alanine scan studies of the endogenous melanocortin peptides α-MSH and

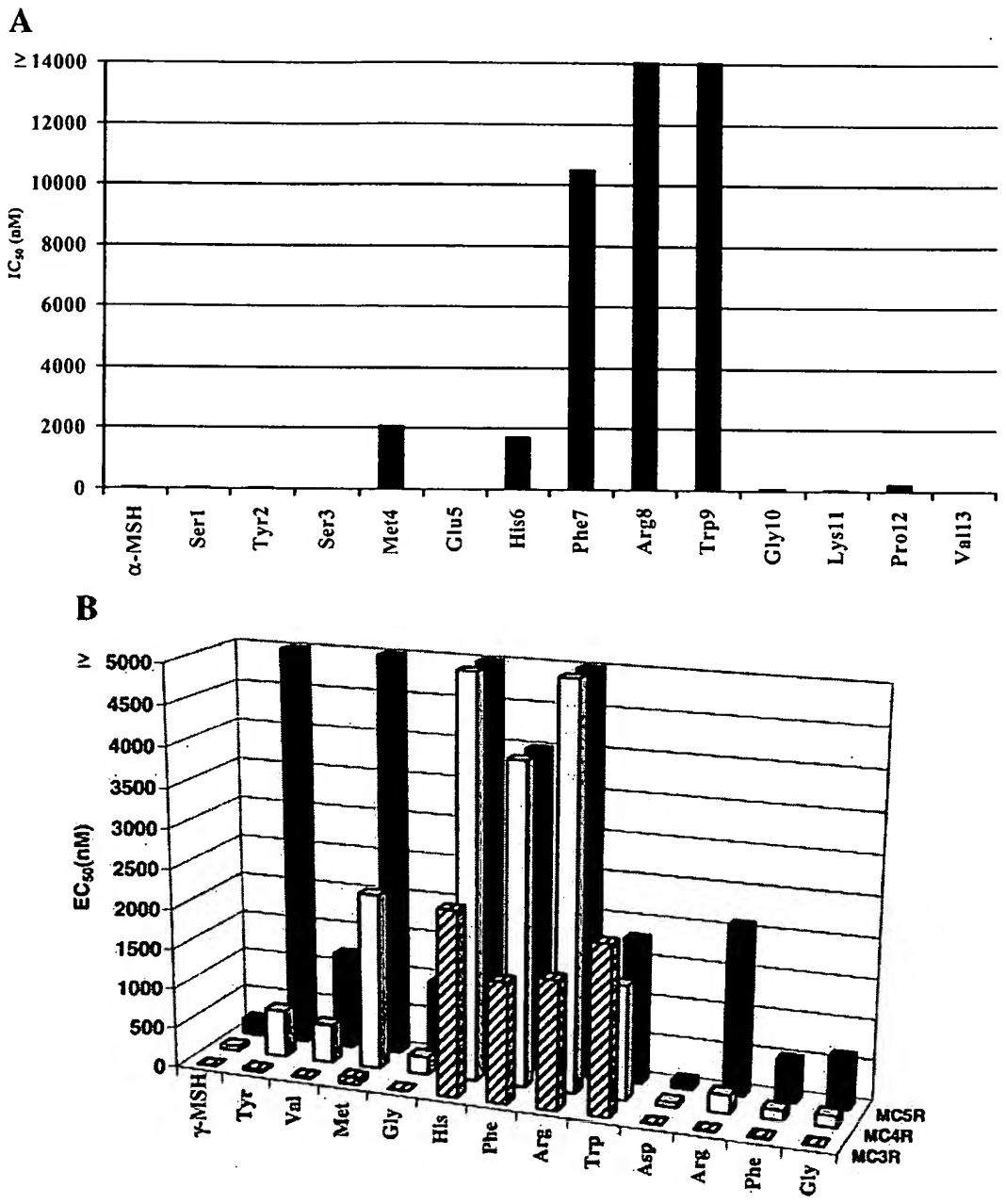


Figure 2. **A**: Graphical representation summarizing the alanine scan of α-MSH. Values indicate the binding affinity of each alanine analogue, as compared with α-MSH, in B16 murine melanoma cells. Data taken from Ref.[82] **B**: Graphical representation summarizing the alanine scan of γ-MSH. EC_{50} values are reported for the cloned human melanocortin MC3 – MC5 receptors. Data taken from Ref.[83]

γ-MSH are illustrated in Figure 2. Discussed below are additional studies that involved replacement each of the core His-Phe-Arg-Trp residues of various melanocortin ligands with alanine.

A. Substitution of His[6]

Histidine has been replaced with alanine in several melanocortin peptide templates, and generally alanine replacement of this residue results in decreased agonist potency and/or binding affinity of the

substituted analogs. When His was replaced by Ala in α-MSH there was a 100-fold decrease in binding affinity at the mouse B16 melanoma cells (putative MC1R), but this analog was only 6-fold less potent than the native hormone in functional activity.[82] When His of the endogenous agonist γ-MSH was replaced with Ala, a 1,600-fold, >90-fold, and >25-fold decreased potency resulted at the hMC3R, hMC4R, and hMC5R, respectively, and Ala substitution of His in the linear NDP-MSH template resulted in a 4-fold decrease in potency at the hMC4R.[80,83] In the linear Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide, when His was replaced with Ala, potency decreased from 58- to 90-fold at the mouse MC1R, MC3R–MC5R.[84] There was a greater reduction in potency when His was completely removed from the tetramer, as the Ac-DPhe-Arg-Trp-NH_2 tripeptide was considerably less potent than the tetrapeptide at the mouse melanocortin receptors.[18] Replacement of the His residue with Ala in the cyclic 23-membered MTII template only resulted in up to a 5-fold decrease in potency at the hMC3R, hMC4R, and hMC5R, whereas removal of His in the [des-His^6]-MTII peptide resulted in complete loss of activity.[85] In the cyclic $COCH_2CH_2CO$-c[His-DPhe-Arg-Trp-Lys]-NH_2 23-membered ring template, His replacement with Ala resulted in interesting pharmacology.[86] There was a slight increase in potency at the hMC3R, however, the peptide was only able to stimulate cAMP production to 19%, indicating a partial agonist. A slight reduction in potency was seen at the hMC4R, but again resulted in a partial agonist with only 35% maximal activity. A complete loss of activity was seen at the hMC5R for the alanine analog. These data combined suggests that the His^6 residue plays a sequence dependent role in the formation of stable ligand–receptor complexes, as evident from the large variation in the activity of the Ala^6 peptide analogs. Some melanocortin ligand templates require the presence of the imidazole ring to retain ligand potency, as demonstrated by the [Ala^6]-γ-MSH analog. Conversely, the Ala^6 analogs of MTII and NDP-MSH were as potent, or nearly equipotent to the parent peptides, suggesting that these two ligand templates can form stable ligand–receptor complexes without assistance from the His imidazole ring. The activity of the $COCH_2CH_2CO$-c[Ala-DPhe-Arg-Trp-Lys]-NH_2 peptide makes consistent interpretation of the His^6 role in melanocortin ligand–receptor interactions even more complex. The potency of this peptide is similar to that of the parent compound the MC3R and MC4R, however, a dramatic reduction in receptor activity resulted from the Ala^6 substitution. This data indicates that the His imidazole ring may not play a large role in formation of the melanocortin ligand–receptor complex, but suggests that the imidazole ring permits the conformational changes to take place within the receptor that are required for signal transduction to occur.

B. Substitution of Phe^7

The phenylalanine residue has been replaced with alanine in several melanocortin peptide templates and generally results in a drastic reduction of potency, or a complete loss of ligand efficacy. When Phe^7 was replaced with Ala in endogenous α-MSH, there was a 200-fold decrease in ligand affinity and a 260-fold reduction in potency in the mouse B16 melanoma cell assay (putative mMC1R).[82] When Ala was substituted for Phe^7 in γ-MSH, the peptide had a 1,500-fold reduction in potency at the hMC3R and the potency was reduced to >4 μM at both the hMC4R and hMC5R.[83] Substitution of the $DPhe^7$ residue with D-alanine in the linear NDP-MSH template, resulted in 1,200-fold decreased ligand potency at the hMC4R. Substitution of alanine for DPhe in the linear Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide resulted in a 1,500-fold decreased potency at the mMC1R and a complete loss of activity at the mMC3R–mMC5R at concentrations up to 100 μM.[87] Replacement of $DPhe^7$ with alanine in the cyclic MTII template reduced the percentage of cAMP accumulation (relative to α-MSH) to 2% at 20 μM, 5% at 10 μM, and 32% at 20 μM at the hMC3R, hMC5R, and hMC4R, respectively.[85] In the cyclic $COCH_2CH_2CO$-c[His-DPhe-Arg-Trp-Lys]-NH_2 template, Phe^7 replacement with Ala resulted in the inability to stimulate the hMC3R–hMC5R at concentrations up to 10 μM.[86] These data clearly illustrate the importance of the phenylalanine residue of the melanocortin agonist templates, indicating that the aromatic phenyl ring significantly contributes to the formation of the stable ligand–

receptor complex. These results are in agreement with the results from *in vitro* melanocortin receptor mutagenesis studies of the MC1R[88–91] and MC4R.[76,80] Melanocortin receptor mutagenesis and three-dimensional homology molecular modeling of the MC1 and MC4 melanocortin receptors indicate that the two receptors contain similar putative ligand–receptor binding pockets consisting of a hydrophobic-aromatic network of Phe receptor residues proposed to interact with the aromatic residues of the melanocortin ligands.

C. Substitution of Arg[8]

Similar to Phe[7], when Arg[8] of melanocortin peptides is replaced with alanine, this modification generally resulted in large decreased ligand potency or the inability to stimulate the melanocortin receptors. When alanine was substituted for Arg[8] in α-MSH, a 2,080-fold reduction in binding and a 100-fold decrease in potency was reported in the mouse B16 melanoma cell assay.[82] Substitution of Ala for Arg[8] in the γ-MSH template resulted in 1,600-fold decreased potency at the hMC3R and the potency was reduced to >5 μM at both the hMC4R and hMC5R.[83] Replacement of Arg[8] with alanine in the linear Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide resulted in decreased potency ranging from 170- to 1,740-fold at the mouse MC1R and MC3R–MC5R.[92] The potency of the cyclic Ala[8]-analog of MTII was reduced between 40- and 280-fold at the hMC3R–hMC5R. In the cyclic $COCH_2CH_2CO$-c[His-DPhe-Arg-Trp-Lys]-NH_2 template, the Ala[8] analog showed no detectable cAMP stimulation at the hMC4R and hMC5R at concentrations up to 10 μM, and was only a weak partial agonist at the hMC3R.[86] Receptor mutagenesis studies of the MC1R[88,90] and MC4R[76,80] have postulated a putative ionic interaction between the positively charged arginine residue of melanocortin peptides and the negatively charged residues located in the TM2 and TM3 regions of the receptors. Figure 3 illustrates the putative ionic interactions between the core melanocortin agonist Arg[8] residue and the acidic residues of the mouse MC4R transmembrane regions. Additionally, it has been suggested that the presence of the two terminal NHs of the arginine side chain, rather than the positive charge, is essential for peptide interaction with the MC1 and MC4 receptors.[93] The results of these alanine scan

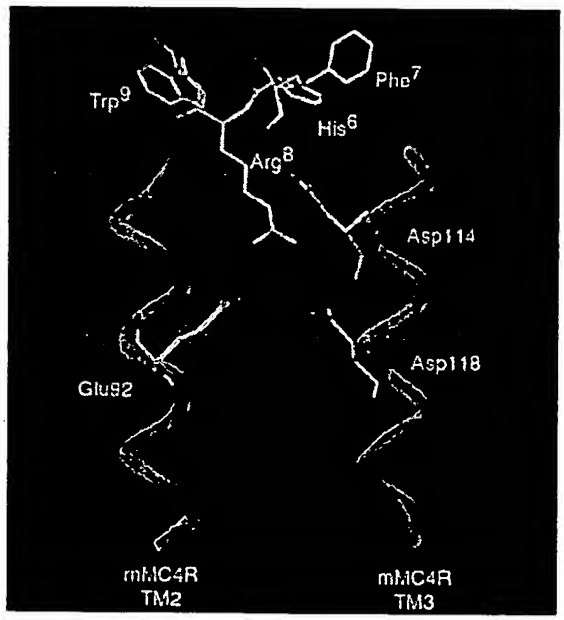


Figure 3. Illustration of the putative ionic interactions the occur between the core melanocortin agonist Arg[8] residue and the acidic residues of the mouse MC4R transmembrane regions. Oxygen atoms are represented in red and nitrogen atoms are represented in yellow for clarity.

studies indicate that the Arg^8 residue of melanocortin peptides, and related peptide analogs, plays an important role in molecular recognition and receptor activation. It is important to note, however, that non-peptide ligands lacking the basic gaunidinyl functionality have been reported for the melanocortin receptors.[94–100] Many of these non-peptide compounds do not have any basic side chain groups that can putatively form an ionic interaction with the receptors, although many aromatic groups are present that can potentially serve as surrogates for the imidazole, phenyl, and indole moieties found in the "core" peptide sequence (see "Non-Peptide Ligands" below). Indeed, highly potent and receptor selective non-peptide agonists that lack any "arginine-like" functionality have been reported for the MC1R[51] and MC4R.[96] The discovery of potent small molecule ligands for the melanocortin receptors that are devoid of basic residues opens debate as to the exact role that the Arg^8 side chain has in receptor stimulation, and may suggest that the spatial orientation of the aromatic groups is more important than the presence of a basic "arginine-like" moiety.

D. Substitution of Trp^9

Substitution of Trp^9 by Ala in the α-MSH template resulted in a 2,000-fold decreased binding affinity and 125-fold decreased potency at the MC1R.[82] The Ala^9 analog of γ-MSH possessed a 26–2,100 fold reduction in potency at the hMC3R–hMC5R and lacked the ability to stimulate the maximal response in the functional bioassay.[83] When Trp^9 is replaced with alanine in the linear Ac-His-DPhe-Arg-Trp-NH_2 template, a complete loss of activity resulted at the mMC3R, however, 220-, 2,540-, and 9,700-fold decreased potency was observed at the mMC1R, mMC4R, and mMC5R, respectively.[101] A larger effect was seen in the cyclic MTII template, as the alanine substituted Trp analog, was only able to generate between 2 and 21% maximum cAMP accumulation (relative to α-MSH).[85] It is evident from these experiments that the aromatic functionality provided by the Trp^9 residue plays a pivotal role in the interaction of the above peptides with the melanocortin receptors.

4. MODIFICATION OF THE CORE His-Phe-Arg-Trp SEQUENCE

Once the minimal sequence essential to retain ligand potency and biological activity has been identified in a lead peptide template, the next step in obtaining important ligand–receptor information is to probe for additional ligand side chain requirements and preferences involved in the formation of stable ligand–receptor complexes.[102] Introduction of stereochemical modifications (D-amino acid scans), functional group modifications, conformational constraints, and topographical constraints to the "core" peptide sequence provide an effective means to investigate these ligand preferences and enhance potency, stability, and receptor selectivity. Modifications mentioned above to the peptide "core" sequence, especially when combined with modern biophysical techniques such as two-dimensional nuclear magnetic resonance (NMR) spectroscopy, may provide insight into the putative "bioactive" conformation of the peptide backbone and may indicate topographical preferences of amino acid side chain moieties. Enzymatic stability can be increased dramatically by introduction of unnatural modifications, as demonstrated with modified melanocortin peptides such as NDP-MSH[103] and MTII.[81,104,105] Insight regarding preferred ligand topography may be gained from these types studies, and may lead to the discovery of analogs with increased potency, receptor selectivity, and *in vivo* duration of action. Discussed below are various modifications that have been made at the melanocortin core tetrapeptide sequence His-DPhe-Arg-Trp.

A. Stereochemical Inversion of Amino Acids

It was noted in early investigations that the biological properties of α-MSH were altered following heat-alkali treatment[106–109] as a result of partial amino acid racemization, and it was hypothesized that synthetic stereostructural analogs may similarly result in enhanced biological activities. Through

the use of high-resolution gas chromatographic methods, it was identified that the Phe^7 residue was the predominant racemized residue and it was speculated that this amino acid may be responsible for the enhanced properties observed for racemized α-MSH activity.[103] This same study yielded the highly potent and "prolonged" acting α-MSH analog, [Nle^4,DPhe^7]-α-MSH (NDP-MSH). Compared to α-MSH, NDP-MSH is enzymatically stable, more potent, and had prolonged biological activity.[103,110] Since the discovery of NDP-MSH, this peptide has been used extensively to characterize the melanocortin receptors and has been the lead compound in many SAR studies.

In recent investigations, each amino acid residue of melanocortin peptides have been replaced with the corresponding stereoisomer and the pharmacological effects of this have been evaluated.[84,87,92,101,111] These studies utilized cloned melanocortin receptors from both human and mouse to evaluate the effects of substitution of L-amino acids with the corresponding D-isomer. Stereochemical inversion generally resulted in decreased, or loss of, agonist functional activity of the melanocortin ligands, with the exception of the DPhe^7 modification. These data are in agreement with previous findings using the classical skin bioassays.[81] The importance of amino acid stereochemistry was illustrated when each of the four amino acids in the Ac-His-Phe-Arg-Trp-NH_2 tetrapeptide were systematically replaced with the corresponding epimer. The inversion of chirality resulted in a reduction in potency for each of the analogs tested at the mouse melanocortin receptors,[84,92,101] with the exception of the DPhe^7 peptide.[87] Greico et al.[111] have replaced each residue in γ-MSH with the D-isomer, which resulted in a similar reduction of potency for all but two of the D-analogs tested at the human receptors. The [DPhe^7]-γ-MSH analog exhibited increased potency as expected, although MC3R versus MC4R selectivity was diminished (ca 50-fold MC3R vs. MC4R selectivity for endogenous γ-MSH). It is interesting to note that substitution of Trp^9 with DTrp^9 increased the potency of γ-MSH at the human MC3R by 17-fold, as well as increasing the MC3R versus MC4R and MC5R selectivity to ca 300- and 250-fold, respectively.

B. Halogenated and Bulky Aromatic Amino Acid Substitutions

Prior to 1995 the melanocortin receptors had mainly been characterized by peptide agonists, such as the highly potent linear peptide NDP-MSH and the cyclic lactam analog MTII (Table II). The cloning of the remaining MC receptors made the need for potent and selective antagonists for *in vitro* and *in vivo* characterization evident, but at that time only a few reports of melanocortin antagonists had been made.[112–115] Using the MTII cyclic template as a starting point, Hruby et al.[116] made various stereoelectronic modifications of the DPhe^7 residue that resulted in some interesting and exciting discoveries. These investigations involved modification of the DPhe^7 residue and were based on previous suggestions that the His^6, Phe^7, Arg^8, and Trp^9 residues are critical for receptor binding and activation.[15,16] The authors postulated that modification of the DPhe^7 residue may disrupt the bioactive conformation of the peptide,[116] preventing signal transduction from occurring, but still permit ligand binding to the "inactive" state of the receptor. Thus, the DPhe^7 amino acid was substituted with a variety of "bulky" aromatic amino acids. This study led to the discovery of two potent and selective (for the MC3R and MC4R) melanocortin receptor antagonists, SHU9119 and

Table II. Sequence Comparison of Common Synthetic Melanocortin Ligands

Peptide	Sequence
NDP-MSH	Ac-Ser-Tyr-Ser-***Nle***-Glu-His-***DPhe***-Arg-Trp-Gly-Lys-Pro-Val-NH_2
MTII	Ac-Nle-cyclo[Asp-His-***DPhe***-Arg-Trp-Lys]-NH_2
SHU9119	Ac-Nle-cyclo[Asp-His-***DNal(2')***-Arg-Trp-Lys]-NH_2
SHU8914	Ac-Nle-cyclo[Asp-His-***(pI)DPhe***-Arg-Trp-Lys]-NH_2

SHU8914. Table II lists the sequences of the cyclic peptide agonist and antagonists, with emphasis of position seven modifications. Substitution with DNal(2′) at the Phe^7 position resulted in agonist activity at the hMC1R and the mMC5R, potent antagonist activity at the hMC3R with partial agonist activity, and potent competitive antagonist activity at the hMC4R (sub nM). Modification of the phenyl ring in the para position resulted in interesting pharmacology. Para substitution of the phenyl ring with either fluorine or chlorine retained agonist activity at all the cloned melanocortin receptors. Replacement of DPhe with D-p-iodophenylalanine [(pI)DPhe] resulted in full agonist activity at the hMC1R, partial agonist activity at the mMC5R, partial agonist as well as potent antagonist activity at the hMC3R and hMC4R. The data from this study led to the hypothesis that bulky aromatic amino acid substitutions at position seven are responsible for differentiating agonist versus antagonist activities of melanocortin ligands.[116]

Since the discovery of SHU9119, there have been various melanocortin peptides synthesized with DNal(2′) at the seven position, and this generally results in ligands with partial agonist and/or antagonist activity.[76,86,87,117–123] Recently, the cloned mouse MC4R was characterized by *in vitro* mutagenesis using several melanocortin based ligands, and many contained naphthyl substituents similar to SHU9119.[76] Table III lists the sequences of the peptides used to characterize the MC4R wild type and mutant receptors. At the wild type MC4R: SHU9005 is a partial agonist as well as a potent antagonist ($pA_2 = 9.7$); [DNal(1′)]-MTII is a partial agonist ($EC_{50} = 0.12$ nM); and [Nal(2′)]-MTII is a full agonist ($EC_{50} = 0.89$ nM) at the mouse MC4R.[117] The mutagenesis data suggested that the phenylalanine receptor residues at positions 254 and 259 of the mMC4R may be responsible for differentiating agonist versus antagonist activity of the melanocortin based antagonist, since mutation of either of these residues to Ser resulted in stimulation of the mMC4R mutants by the antagonists.[76] Using the naphthyl based peptides to characterize the wild type and mutant mMC4R has led to a revision of the original hypothesis regarding the ability of bulky aromatic groups at position seven to impart antagonistic activity to melanocortin peptides. This mMC4R receptor mutagenesis experimental evidence suggests that not only is the bulky aromatic moiety required for antagonism of the MC4R, but the stereochemistry of the α-carbon and the position of the naphthyl ring (1′ vs. 2′) are also important.[76] The structures of naphthylalanine amino acids emphasizing the stereochemistry and ring position are shown in Figure 4.

Characterization of Phe254Ser and Phe259Ser MC4R mutants with the Ac-His-DNal(2′)-Arg-Trp-NH_2 tetrapeptide further implicates these two phenylalanine residues in differentiating agonist versus antagonist activity of melanocortin based ligands.[87] The peptide is a competitive antagonist at the wild type MC4R ($pA_2 = 7.78$), however, the peptide is converted into an agonist at the Phe254Ser and Phe259Ser mMC4 mutant receptors.[87] The above revised hypothesis regarding the importance of both stereochemistry and ring position of naphthylalanine residues in achieving peptides with antagonist activity is further supported in this latter report, since the only tetrapeptide derivative that resulted in an MC4R antagonist contained a DNal(2′) naphthyl moiety [DNal(1′) derivative is an agonist, Nal(1′) and Nal(2′) analogs are inactive at the mMC4R].[87]

Table III. Melanocortin Peptides Used to Characterize Mouse MC4R Mutant Receptors

Peptide	Sequence
MTII	Ac-Nle-cyclo[Asp-His-***DPhe***-Arg-Trp-Lys]-NH_2
SHU9119	Ac-Nle-cyclo[Asp-His-***DNal(2')***-Arg-Trp-Lys]-NH_2
SHU9005	Ac-Ser-Tyr-Ser-Nle-Glu-His-***(pI)DPhe***-Arg-Trp-Gly-Lys-Pro-Val-NH_2
[DNal(1')]-MTII	Ac-Nle-cyclo[Asp-His-***DNal(1')***-Arg-Trp-Lys]-NH_2
[Nal(2')]-MTII	Ac-Nle-cyclo[Asp-His-***Nal(2')***-Arg-Trp-Lys]-NH_2

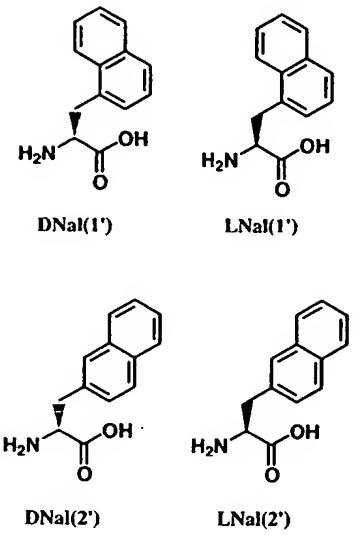


Figure 4. Structure of naphthylalanine amino acids. The amino acids only differ as a result of stereochemistry or position of ring substitution.

It has been suggested that the topographical orientation of the SHU9119 arginine residue is modified by the presence of an adjacent naphthalene ring, as compared to the topography of the arginine side chain of MTII.[76] This hypothesis is the result of the different pharmacological profiles observed from MTII, SHU9119, [DNal(1′)]-MTII, and [Nal(2′)]-MTII at mutant mouse MC4 receptors.[76] Several studies have suggested that acidic residues present in TM2 and TM3 of the melanocortin receptors interact with the basic arginine residue of melanocortin ligands.[76,80,88,91,124] The putative arginine–MC4R interactions are illustrated in Figure 3. Using site-directed mutagenesis, the acidic residues in TM2 and TM3 of the MC4R were systematically exchanged with basic residues to test this hypothesis.[76,80] The pharmacological results varied for each of the naphthyl-MTII derivatives tested at the MC4R mutants. At the Asp114Arg mutant mMC4 receptor, there was a 30-fold difference between [DNal(1′)]-MTII and [Nal(2′)]-MTII, and a 17-fold difference between MTII and [Nal(2′)]-MTII. Additionally, at the Glu92Lys mMC4R mutant, SHU9119 only had a 3-fold decrease in binding affinity whereas MTII had a 74-fold decrease. Furthermore, SHU9119 was the only peptide that retained binding ability to the Asp118Lys mMC4R mutant. These data indicate that the different ligands, each with a different aromatic functionality at the seven position, potentially interact with the mMC4R Asp114, Asp118, and Glu92 receptor residues in a differential manner. These data suggest that the topographical arrangement of the arginine side chain in three-dimensional space may be different in each of the peptides, and thus results in different interactions with MC4R acidic residues. In this regard, the interactions may be responsible for the different pharmacological profiles of the naphthyl ligands and the antagonistic activity of SHU9119 at the MC4R. However, the hypothesis that the aromatic ring structures are indeed modifying the arginine side chain in three-dimensional space has yet to be verified experimentally. In recent efforts to discern the molecular determinants responsible for SHU9119 antagonism of the hMC4R, Yang et al.[124] were able to convert SHU9119 from an antagonist to an agonist by modification of the TM3 region of the receptor. This report provided experimental data to suggest that Leu133 of the human MC4R plays a key role in SHU9119 antagonist activity.[124]

Substitution of D-p-iodophenylalanine [(pI)DPhe] for D-phenylalanine at position seven has also been reported to modify the pharmacological activity of melanocortin peptides. The peptides SHU9005 (Table III)[76] and SHU8914 (Table II)[116] both contain (pI)DPhe at the seven position and are partial agonists with potent antagonist activities at the MC3R and MC4R. These data support the

hypothesis that substitution of the Phe^7 residue with bulky aromatic amino acids can result in antagonists analogs.[116] Interestingly, the tetrapeptide Ac-His-(pI)DPhe-Arg-Trp-NH_2 (JRH 322-18) is a full nM agonist at the mMC1 and mMC5 receptors, a mMC3R partial agonist with potent antagonist activity ($pA_2 = 7.25$, $Ki = 56$ nM), but unexpectedly, is a full and potent agonist at the mMC4R ($EC_{50} = 25$ nM).[87] To determine if the observed pharmacology was species specific, JRH 322-18 was tested at the human MC4R and similar agonist pharmacology was observed ($EC_{50} = 5$ nM). This ligand possesses novel melanocortin receptor pharmacology, compared to previously reported (pI)DPhe based peptides, and is potentially useful for *in vivo* studies to differentiate MC3R versus MC4R physiological roles in animal models, such as primates, where "knock-out" animals are not viable options.

It has been suggested that large stereoelectronic modifications of the message sequence His-Phe-Arg-Trp of melanocortin ligands may produce antagonists for the MC3R and MC4R.[116] This appears to be true only for bulky aromatic amino acids substituted for the Phe^7 residue, as similar substitutions at the 6, 8, and 9 residues have failed to generate any analogs with antagonist activity.[84,92,101] Naphthylalanine substitutions for His^6 and Arg^8 are not well tolerated and generally results in analogs lacking any agonist or antagonist activity,[84,92] however, substitutions of the Trp^9 residue are generally more tolerated.[101] Indeed, naphthylalanine substitutions have been made for Trp^9 with no decrease in ligand potency.[101,117] These data suggest that the chemically reactive Trp indole side chain may be replaced with the non-reactive naphthyl moiety in the design of peptide and non-peptide melanocortin receptor ligands, as long as the naphthyl ring is in the correct orientation (1′ vs. 2′).

C. Constrained Amino Acid Substitutions

One means of restricting the conformational flexibility of a peptide backbone is the use of constrained amino acids.[125,126] Structures of constrained amino acids commonly used to restrict conformational flexibility of the peptide backbone are illustrated in Figure 5. This constrained amino acid strategy has led to the discovery of peptides that show increased binding affinity, potency, and selectivity towards one or more of the melanocortin receptors.[125] Identification of an amino acid constraint that improves ligand potency and selectivity can provide a valuable tool to aid in the development of three-dimensional pharmacophore models of melanocortin peptides, as well as produce novel ligands to aid in the pharmacological and physiological characterization of the melanocortin receptors.

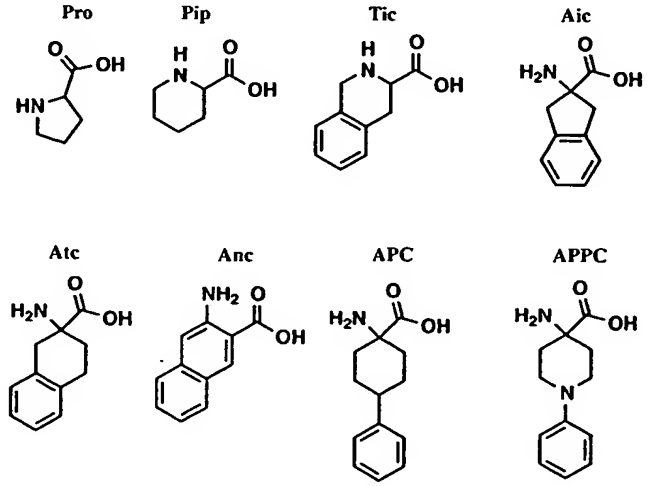
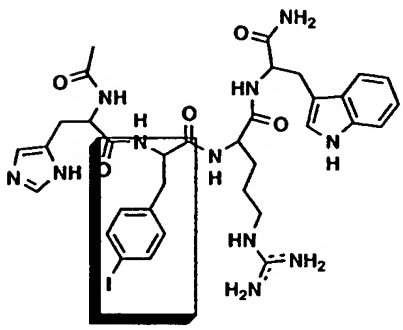
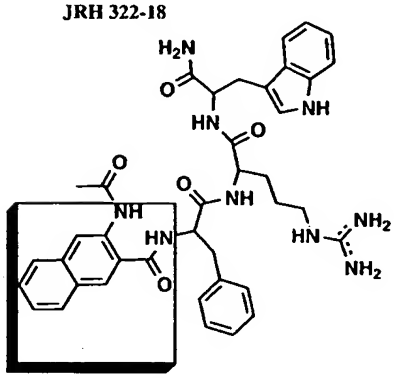


Figure 5. Common constrained amino acids used to restrict conformational flexibility of peptide residues.



JRH 322-18



JRH 420-12

Figure 6. Novel melanocortin tetrapeptides JRH420-12 and JRH322-18. These peptides demonstrate MC4R versus MC3R selectivity. Residues with modified amino acids are highlighted.

Proline has been used to replace histidine in the cyclic MTII[127] and SHU9119[119,128] templates, and the results of these studies suggest that incorporation of amino acids in the His^6 position that restrict conformational freedom of the peptide may lead to increased melanocortin receptor agonist selectivity. Proline and the proline-like 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and D-Tic were incorporated into the linear Ac-His-DPhe-Arg-Trp-NH_2 peptide at position six, but this resulted in decreased potency and no increased receptor selectivity at the cloned mouse melanocortin

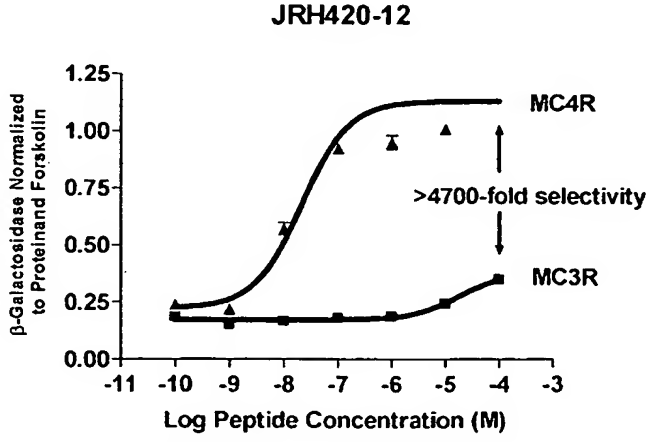


Figure 7. Agonist dose response curves of JRH 420-12 at the mouse MC3 and MC4 receptors, illustrating the MC4R versus MC3R selectivity.

receptors.[84] These later data suggest that the imidazole ring of His^6 is required in the relatively short sequence of the tetrapeptide to retain potency, and/or that the added conformational rigidity that these constrained amino acids impart to the tetrapeptide is unfavorable at the melanocortin receptors.

Recently, additional experimental evidence has emerged that strongly indicates that modification of the His^6 position can increase MC4R versus MC3R selectivity.[84,128,129] Danho et al.[129] evaluated a series of cyclic peptides based on the penta-cyclo-[Asp^5,DPhe^7,Lys^{10}]-α-MSH_{5-10}-NH_2 template modified at the His^6 position. The peptides were pharmacologically characterized for agonist activity at the cloned human MC1, MC3–MC5 receptors. Histidine substitutions were made with 2-amino-tetrahydro-2-napthyl carboxylic acid (Atc), and the novel amino acids 1-amino-4-phenylcyclohexane-carboxylic acid (APC) and 4-aminophenylpiperidine-4-carboxylic acid (APPC).[129] When His^6 was replaced in the cyclic analogs with conformationally constrained amino acids, this resulted in several highly selective agonist peptides for the hMC4R. Similar results were obtained when His^6 was replaced with Atc in the linear Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide.[84] These data indicate that MC4R selectivity can be achieved by incorporating conformationally constrained amino acids in cyclic peptides, and when utilized in more flexible linear peptides. These observations that constrained amino acids can be substituted for His^6 in small, flexible linear peptides encouraged our lab to search for additional conformationally constrained amino acids with aromatic character to replace histidine.[84] This search led to the discovery of JRH 420-12, a highly potent and MC4R versus MC3R selective agonist (>4,700-fold selectivity). The structures of JRH 322-18 and JRH 420-12, novel tetrapeptides for the melanocortin receptors, are illustrated in Figure 6. In the JRH 420-12 melanocortin agonist, His^6 has been replaced with the unusual amino acid amino-2-napthyl carboxylic acid (Anc), a β-amino acid based on the bicyclic naphthyl moiety. JRH 420-12 resulted in slight agonist activity at the MC3R (<50% maximal stimulation at 100 μM), but was a full agonist at the MC4R ($EC_{50} = 21$ nM). Figure 7 illustrates the agonist dose response curves of JRH 420-12 at the mouse MC3 and MC4 receptors, illustrating the MC4R versus MC3R selectivity. It should be noted that JRH 420-12 was a very weak antagonist at the MC3R ($pA_2 = 5.6$, Ki = 2.5 μM). These above data strongly support the hypothesis that the His^6 position is a critical position[84,119,129,130] for the identification of MC4 versus MC3 receptor selective agonist peptides.

D. β-Methyl Amino Acid Substitutions

Knowledge about the bioactive conformation of a ligand may aid in the rational design of potent and selective compounds. This includes knowledge about backbone (φ, Ψ) torsion angles and the preferred Cα-Cβ (χ_1) torsion angles (see Refs.[125,126] for detailed reviews on this subject). Information about the backbone φ, Ψ torsion angles may be obtained from studies designed to introduce global constraints into melanocortin ligands, such as side chain to side chain cyclization. The side chain topography of amino acids [referred to as chi (χ) space] is another important conformational parameter to consider when designing ligands with specific biological activity. Figure 8 provides pictorial definitions of φ, Ψ, and χ torsion angles. Introduction of a methyl group at the side chain β-carbon of aromatic amino acids should reduce the rotational freedom about the χ_1 torsion angle, and thus limit the available topographies of the side chain to either the gauche(−), trans, or gauche(+) conformation.[131] Figure 9 shows Newman projections of the three low energy staggered conformations of L-amino acids. Incorporation of β-substituted amino acids has proven to be a successful strategy to determine the topographical preferences of a variety of biologically active peptides.[126,132–138] A second chiral center is created by β-substitution and four diastereomers result when considering both L- and D-amino acids (2S,3S; 2S,3R; 2R,3S; 2R,3R). The premise behind β-substitution is to bias the χ_1 torsion angle into one major low energy rotamer population. However, it appears that introduction of a single β-methyl substitution often discards one rotamer population while leaving two predominant populations, dependant upon the peptide template used.[125] Following characterization of the β-substituted ligands in the appropriate bioassay, it can be determined which

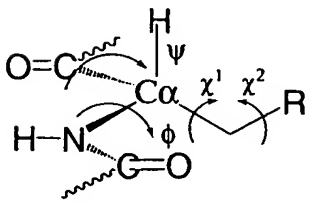


Figure 8. Pictorial definitions of φ, Ψ, and χ torsion angles of the peptide backbone.

diastereoisomeric analogs result in decreased or increased binding affinity and/or potency, with the caveat that introduction of the methyl group may interfere with ligand–receptor interactions.

Introduction of a diastereoisomeric amino acid may affect affinity and potency, as well as have a significant affect on the biological phenomenon of prolonged (residual) activity.[131] Prolongation has been previously defined as: (a) not prolonged if skin darkening is less than 50% of the maximal response 60 min following ligand removal from the assay medium; (b) prolonged if skin darkening remains greater than 50% of the maximal response 60 min following ligand removal from the assay medium; or (c) superprolonged if skin darkening is greater than 90% of the maximal response for longer than 60 min following ligand removal from the assay medium.[139] Figure 10 provides graphical definitions of superprolongation, prolongation, and no prolongation using the classical skin bioassays. The observed residual activity has been attributed to slow dissociation rates of the β-substituted analogs from the receptors.[110,131] Once a diastereoisomeric analog is found to possess differential activities, such as potency and prolongation, homonuclear and heteronuclear NMR studies can be employed to determine the topographical orientation of the β-substituted side chain (predominant χ_1 rotomer population). Incorporation of β-substituted amino acids has proven to be a successful strategy to determine the topographical preferences of a variety of biologically active peptides.[126,132–138]

Introduction of topographical constraints into the cyclic α-MSH analog, MTII, results in differential potencies, dissociation rates, and prolonged activities at the melanocortin receptors. MTII analogs modified at the Trp^9 position with β-methyltryptophan have been evaluated using the classical frog and lizard skin bioassays, and at the cloned human MC1R.[131,140] The 2S,3S analog had the highest affinity and potency out of the four MTII derivatives at the cloned human MC1R, and also exhibited the slowest dissociation rate from the MC1R (25% slower than MTII). The 2S,3S-MTII analog presumably has a conformation that allows for "tighter" binding to the MC1R than the other three analogs, and thus the stronger receptor–ligand interactions result in slower dissociation of the ligand from the receptor. In the frog skin bioassay, the 2R,3R-MTII analog exhibited superprolonged activity, whereas in the lizard skin bioassay three of the four β-methyltryptophan derivatives possessed prolonged biological activity (the 2S,3R analog showed no prolonged activity). Data from NMR studies suggest that the preferred side chain populations of the Trp^9 residue modifications of MTII were gauche(−) in the 2S,3S, trans in the 2S,3R, and gauche(+) in the 2R,3R and 2R,3S analogs.[125,141] The above studies indicate that topographical constraints, such as β-methyl amino acids, can provide a useful tool to determine the preferred side chain orientation, and this data may be

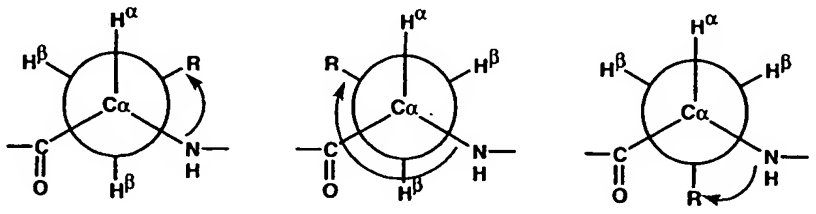


Figure 9. Newman projections of the three low energy staggered conformations of L-amino acids.

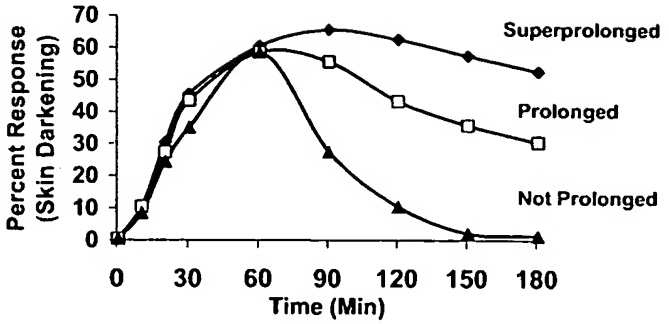


Figure 10. Graphical definitions of superprolongation, prolongation, and no prolongation in the classical melanocortin skin bioassays.

used to design of ligands with potent and selective activity. These data illustrate the topographical recognition differences between the three MC1 receptors (frog, lizard, and cloned human) and emphasize that previous SAR studies using the classical frog and lizard skin bioassays may not correlate with the SAR studies using the cloned human melanocortin receptors.

5. N-TERMINAL MODIFICATIONS

Recently detailed SAR studies of the melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 at the mouse melanocortin receptors have been reported.[84,87,92,101] In these studies over 75 tetrapeptides were synthesized with modifications at each of the 4 residues, and were pharmacologically characterized at the mouse MC1, MC3–MC5 receptors. These studies led to the discovery of potent ligands for the melanocortin receptors that exhibit novel pharmacology. Several trends emerged from the SAR studies, such as stereochemical preferences at each position, preferential side chain lengths, and positions responsible for receptor selectivity. Following the SAR studies that involved modifications at 6, 7, 8, and 9 positions, it was decided to investigate modifications at the N-terminus and the effects on ligand potency and selectivity.[142,143] α-MSH contains a N-terminal acetyl group as a result of endogenous posttranslational processing, which is believed to enhance enzymatic stability of the peptide.[10,105] The majority of melanocortin peptide SAR studies have included the N-terminal acetyl group, however, little is known regarding what affect additional N-terminal moieties will have on peptide activity. The addition of fatty acid conjugates,[144,145] biotin,[146] and chlorotriazinylamino-fluorescein[147] to the N-terminus of melanocortin peptides have been made, and resulted in enhanced or decreased potencies in the classical skin, lizard skin, and tyrosinase melanocortin bioassays, dependent on the type of modification made. In a more recent report, MTII analogs modified at the N-terminus with proline, cyclopentyl, and other various acyl groups were found to enhance receptor selectivity.[127] Additionally, two linear pentapeptides that contained a butyryl moiety at the N-terminus were reported to be MC4R versus MC3R selective agonist and antagonist.[120] In our efforts to ascertain the affects of N-terminal modification on biological activity, 25 tetrapeptides were synthesized using the His-DPhe-Arg-Trp-NH_2 template modified at the N-terminus with linear, cyclic, and aromatic acyl moieties.[142,143] Structures of acyl groups used to modify the N-terminus of the melanocortin tetrapeptide agonist are shown in Figure 11. The peptides were pharmacologically characterized for agonist activity at the MC1R, MC3R–MC5R and assessed for increased potency and enhanced receptor selectivity.

Modifications of the N-terminus that consisted of linear aliphatic chains increased ligand potency as the chain length was extended. The tetrapeptide modified at the N-terminus with the octanoyl group had the largest potency enhancement out of all the alkyl chains tested (70-, 105-, and 110-fold

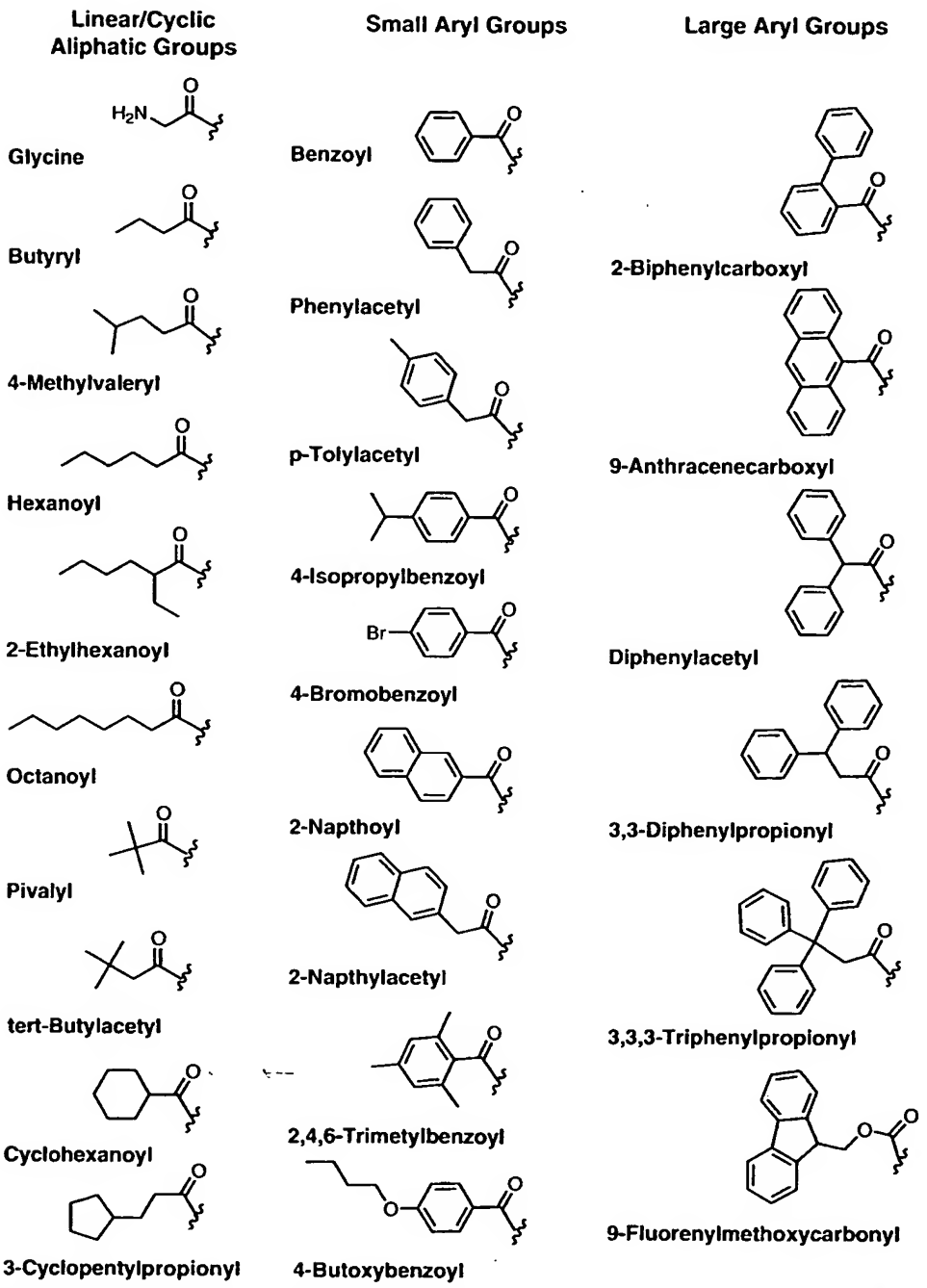


Figure 11. Various linear, cyclic, and aromatic acyl moieties used to modify the N-terminus of the melanocortin His-dPhe-Arg-Trp-NH_2 tetrapeptide.

increased potency at the MC4R, MC3R, and MC1R, respectively, and only 8-fold at the MC5R). This *N*-octanoyl containing tetrapeptide possesses an agonist $EC_{50} = 0.38$ at the MC4R, which is equivalent (within experimental error) to the highly potent tridecapeptide NDP-MSH, and 14-fold more potent than the endogenous melanocortin peptide α-MSH. This data suggested that increasing the alkyl chain length past the eight carbon chain of the octanoyl group may increase ligand potency to

an even greater extent, although this has not been verified experimentally. The increase in potency observed from addition of aliphatic chains to the tetrapeptides may be attributed to an increase in hydrophobic ligand–receptor interactions, since the melanocortin receptors putatively contain a hydrophobic binding pocket.[76,80,88,91] An alternative explanation may be that introduction of hydrophobic acyl groups to the N-terminus enhance peptide–lipid interactions along the membrane–liquid interface, thereby increasing availability of the ligand to the lipid bilayer.[148–153] One might envision the covalently linked fatty acid as an anchor to the membrane surface, which may serve to concentrate the peptide in a vicinity closer to the membrane embedded receptor in a conformation suited for ligand–receptor interactions.[154]

Modification at the N-terminus of the His-DPhe-Arg-Trp-NH_2 peptide with aromatic moieties resulted in analogs with increased potency as well as increased receptor selectivity. Addition of a p-tolylacetyl group to the N-terminus led to a 30-fold increase in potency at the mMC4R ($EC_{50} = 0.89$ nM), and addition of a 2-naphthylacetyl group enhanced ligand potency between 10- and 30-fold at all the melanocortin receptors characterized. Knittel et al.[155] have reported similar results at the human MC1R, MC3R, and MC4R when they modified the N-terminus of the His-DPhe-Arg-Trp-NH_2 tetrapeptide with additional aryl moieties, such as $Ph(CH_2)CO$, $Ph(CH_2)_2CO$, and $Ph(CH_2)_3CO$. The increase in potency of the tetrapeptide analogs does not appear to be species specific, since N-terminal modifications with structurally related analogs augmented potency at both the cloned mouse and cloned human melanocortin receptors.[142,155] Interestingly, introduction of larger multi-ring aromatic functionalities such as biphenyl or triphenyl groups increased selectivity at certain melanocortin receptors. The 2-biphenylcarboxyl group modification resulted in 110-fold MC4R versus MC3R selectivity for the two centrally located receptors. When 3,3,3-triphenylpropionyl group was introduced at the N-terminus, this resulted in a peptide selective for the MC5R by greater than 100-fold over the remaining melanocortin receptors tested. This latter peptide may serve as a useful tool for *in vivo* characterization of the MC5R, considering few reports have been made regarding ligands selective for this receptor.

These data indicate that potency of relatively short peptides can be significantly enhanced by simple addition of hydrophobic linear and cyclic acyl groups to the N-terminus. These types of modifications may also enhance additional properties desirable for compounds intended for *in vivo* application. Modification of the N-terminus with acyl moieties can increase enzymatic stability, and thus provide a potential means of prolonging the duration of action of the parent peptide.[152,153,156] Introduction of a hydrophobic acyl group is also an effective means of increasing lipophilicity, and thus may enhance peptide interactions with receptors located in cellular membranes and penetration across biological barriers.[149,152,153,157] These data suggest that modification of the His-DPhe-Arg-Trp-NH_2 peptide with additional multicyclic aromatic ring systems may further increase melanocortin receptor selectivity.

6. CYCLIC MELANOCORTIN LIGANDS

As is the case for many linear peptides, α-MSH is rapidly degraded by proteolytic enzymes, and thus has a short duration of action because of clearance from systemic circulation.[104,105] The concept of side chain to side chain cyclization of melanocortin peptides to increase potency and to prolong activity has been applied over two decades ago since the design of Ac-[Cys^4,Cys^{10}]-α-MSH,[158] which contains a disulfide bridge between Cys^4 and Cys^{10} amino acids and resulted in enhanced biological properties. This cyclic disulfide α-MSH analog was up to 1,000-times more potent in the frog skin bioassay, than α-MSH, but was not particularly potent in subsequent evaluations using mammalian bioassays. Sawyer et al. suggested that cyclization constrains the analog in a conformation favorable for peptide–receptor interactions, the "bioactive" conformation, which likely consisted of a reverse β-turn. Recently there has been renewed interest in using disulfide α-MSH analogs to help rigidify

conformational flexibility that has led to the discovery of fairly potent and selective melanocortin ligands.[122,123,159] For instance, Schioth et al. have reported potent and selective disulfide peptide antagonists for the MC4R that increase food intake following intracerebroventricularly (i.c.v.) as well as peripheral administration.[160,161] Molecular mechanics calculations and molecular dynamic simulations were used in the development of two benchmark melanocortin peptides based on side chain to side chain lactam cyclization, MTII and SHU9119 (Table II).[16,116,162] In the molecular dynamics studies, three important observations were made regarding the structure of α-MSH and NDP-MSH. First, both peptides rapidly adopted folded conformations that placed the aromatic His^6-Phe^7(or $DPhe^7$)-Trp^9 residues on the same face of the peptide in β-turn conformation. Second, the hydrophilic Glu^5, Arg^8, and Lys^{11} residues were oriented on the face of the peptide opposite the aromatic groups. Third, although Glu^5 and Lys^{11} were in close proximity to one another, the charged groups were not close enough to form a strong ionic interaction. Al-Obeidi et al. reasoned that if the Lys residue was substituted at the Gly^{10} position, then strong interactions would be more probable between the Glu and Lys side chains. When molecular dynamics simulations were performed on the linear Ac-[Nle^4,$DPhe^7$,Lys^{10}]-α-MSH_{4-10}-NH_2 and the Ac-[Nle^4,Asp^5,$DPhe^7$,Lys^{10}]-α-MSH_{4-10}-NH_2 analogs, a folded configuration was adopted in which the side chain carboxylate group of either Asp or Glu and the side chain amino group of Lys were in a close proximity and a putative reverse turn occurred around the His-DPhe-Arg-Trp residues. Once this observation was made for the linear peptides, the corresponding lactam cyclized MTII and MTII-like peptides were synthesized and were found to be highly potent and exhibited prolonged activity.[163]

MTII and SHU9119 have been used extensively for *in vitro* and *in vivo* characterization of the melanocortin receptors, however, the compounds do not selectively bind to the different melanocortin receptor isoforms.[116] Identification of both agonists and antagonists that are selective for specific receptor isoforms, and understanding the structural and conformational characteristics that might lead to receptor selectivity are a current challenge in peptide and peptidomimetic research. Additional cyclic templates have more recently been utilized to restrict conformational flexibility of melanocortin peptides, and some are reported to enhance receptor selectivity.[86,118,139,164–166] The novel cyclic peptide (O)C-CH_2-CH_2-C(O)-c-[His-DPhe-Arg-Trp-Lys]-NH_2, a 23-membered agonist cyclized from the N-terminus and the ε-amino group of lysine via a succinic linker, shows around 40-fold functional selectivity of the hMC4R compared to the hMC3R.[86,165] The 21-membered cyclic agonist $NH(CH_2)_2C(O)$-c-[His-DPhe-Arg-Trp-Glu]-NH_2 is 90- and 3,400-fold more selective at the hMC4R compared to the hMC3R and hMC5R, respectively.[165] Additionally, cyclic peptide antagonists selective for the hMC3R and hMC4R have been reported. The peptide (O)C-C_6H_4-C(O)-c-[His-DNal(2′)-Arg-Trp-Lys]-NH_2 was found to be a potent antagonist at the MC4R and a partial agonist at the MC3R and MC5R, while the peptide (O)C-$(CH_2)_3$-C(O)-c-[His-DNal(2′)-Arg-Trp-Lys]-NH_2 was a potent antagonist at the hMC3R and was a partial agonist at the hMC4R and hMC5R.[86] Likewise, the cyclic peptide (O)C-CH_2-CH_2-C(O)-c-[DNal(2′)-Arg-Trp-Lys]-NH_2 was reported to be a potent antagonist 125-fold selective of the hMC4R compared to the hMC3R.[118] Introduction of a cyclic constraint may restrict the flexibility of a lead peptide and has proven to be an effective means of generating ligands with enhanced potency, receptor selectivity, and enzymatic stability. However, discovery of a cyclic template alone does not provide much insight into the overall conformational properties responsible for the enhanced activities. Biophysical methods, such as ^{1}H-NMR analysis and modeling studies, can aid in understanding the conformational properties responsible for the biological activity of the modified peptide.

7. ^{1}H-NMR AND CONFORMATIONAL ANALYSIS

Many endogenous peptides are relatively small and often conformationally flexible linear molecules, which makes correlating a "bioactive" conformation with functional activity challenging. The

rationale behind using cyclic constraints is the hypothesis that flexible peptides have many accessible backbone and side chain conformations, and thus in solution the bioactive conformation may only be present in low concentrations.[167] Introduction of a proper cyclic restraint may rigidify the peptide backbone and thus aid in the biophysical analysis and construction of a conformational model, since spectroscopic analysis of a flexible peptide with many conformational states may not provide the actual conformational parameters of one conformation but rather an average of many conformational populations of the peptide.[167]

It was noted in some of the initial studies on α-MSH that potency could be increased when DPhe^7 was substituted for the Phe^7 residue.[103] This observation led to a proposed conformation of α-MSH that consisted of a reverse turn around the Phe^7 residue, since D-amino acids are known to stabilize reverse turn conformations.[168–170] The side chain cyclic analog Ac-[Cys^4,Cys^{10}]-α-MSH was designed to stabilize the bioactive conformation and to test the hypothesis that a reverse turn existed in the peptide.[158] Molecular dynamics studies revealed that a reverse turn was observed in the cyclic analog, as expected. There have been many studies designed to determine the bioactive conformation of α-MSH since the study of Sawyer et al., and the majority of these studies have provided additional experimental evidence to suggest a reverse turn occurs around the core His-Phe-Arg-Trp sequence.[171–178] Studies by Sugg et al.[173] have suggested a bioactive conformational model that contains a β-turn around the core tetrapeptide sequence, which is stabilized by the presence of the DPhe^7 residue, with the His, DPhe, and Trp side chains in close proximity on one surface of the peptide and the Arg side chain on the opposite surface of the molecule. These initial observations related conformation of melanocortin peptides with activity, which led to the design of the highly potent and enzymatically stabile cyclic analog of α-MSH, Ac-Nle-c[Asp^6,DPhe^7,Lys^{10}]-MSH$_{4-10}$]-NH$_2$ (MTII).[163] MTII is a 23-membered ring formed from lactam cyclization through the Lys and Asp side chains. NMR and quenched molecular dynamics studies on MTII indicated that a type II β-turn occurs in the His-DPhe-Arg-Trp region, as compared with a type III β-turn of the linear analog (see Refs.[168–170] for a complete overview of turn structures in peptides and proteins). As previously suggested,[173] this latter study also indicated that the His, DPhe, and Trp side chains are located on one surface of the peptide whereas the Arg side chain was on the opposite face.[174] Theoretical studies of Prabhu et al.[171,172] provide additional evidence to support a bioactive conformation of melanocortin peptides that consist of a stable β-turn. These above data indicate that the solution conformation involves a β-turn around the core His-DPhe-Arg-Trp region of the melanocortin peptides and that the three hydrophobic aromatic rings are in a stacked orientation opposite the hydrophilic side chain of arginine. However, recent NMR and conformational analyses suggest that the DPhe and Trp side chains are not stacked on the surface opposite side chain of arginine.[85,177] Indeed, Elipe et al.[177] have argued that the arginine side chain is oriented in close proximity of the naphthyl ring in the cyclic analog (O)C-CH_2-CH_2-C(O)-c-[DNal(2′)-Arg-Trp-Lys]-NH_2, suggesting a bioactive conformation (at least at the MC4R) that consist of a "V" shape in the core His^6-DNal(2′)^7-Arg^8-Trp^9 region with the arginine side chain oriented between the aromatic rings of Nal and Trp. These above data illustrate how cyclization of a peptide can rigidify the structure and aid in conformational analysis. These data also support a bioactive conformational model for melanocortin peptides that consist of a reverse turn centered around the core His-Phe-Arg-Trp motif, however, the exact nature of the turn and the orientation of the side chains are still debated.

8. NON-PEPTIDE MELANOCORTIN LIGANDS

Considerable effort has gone into the design and development of ligands for the melanocortin receptors with properties not present in the endogenous peptides, such as improved potency, receptor selectivity, and bioavailability. Towards this end, substantial progress has been made in the development of non-peptide molecules for the melanocortin receptors. One of the first reported non-

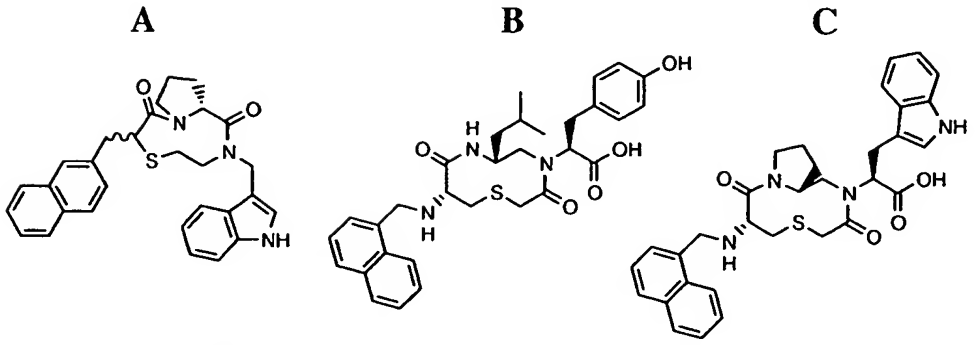
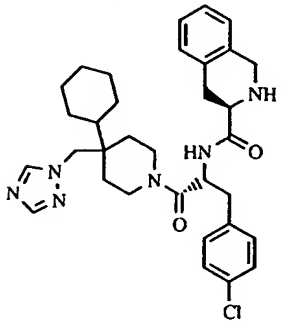


Figure 12. Structures of non-peptide compounds based on the β-turn that activate the MC1R. These melanocortin agonists lack a basic residue to mimic the Arg^8 residue common to melanocortin peptide ligands. Functional activity values are for the MC1R.

peptide ligands for the melanocortin receptors were based upon the β-turn.[95] The β-turn mimetics were based on the Phe-Arg-Trp peptidyl side chains that represent the putative pharmacophore of melanocortin peptides. A library of 951 β-turn compounds was screened at the MC1R for agonist activity and several compounds resulted in receptor stimulation above basal level. Surprisingly, the most potent compound identified (Fig. 12) did not contain a basic group capable of forming an ionic interaction with the acidic receptor residues located in the putative MC1R binding pocket. In a concurrent study, Heizmann et al. screened a large combinatorial library of 328,509 oligo-*N*-substituted glycine trimers (peptoids) and found several compounds with affinity to the MC1R, however, all peptoids identified contained a basic residue. Peptoids with affinity to the MC1R all shared the same structural feature of aromatic residue-basic residue-aromatic residue.[179] In a subsequent study, a novel thioether cyclized scaffold was used to mimic the β-turn.[94] A series of 19 compounds were screened for agonist activity at the mouse melanocortin receptors. Several compounds were identified with agonist activity, and three of the identified compounds were completely devoid of a basic residue capable of mimicking the Arg^8 residue of melanocortin peptides. Examples of non-peptide melanocortin agonists based on the β-turn, that lack a basic residue, are shown in Figure 12. These studies provided some of the first non-peptide ligands for the melanocortin receptors and provided experimental evidence to support a bioactive conformation consisting of a β-turn. These data also called into question the importance of the Arg^8 residue in receptor activation, and suggested that the spatial arrangement of hydrophobic side chains may be more important than the presence of an "arginine-like" basic residue.

In addition to the above studies, several groups have recently reported the design and synthesis of novel non-peptide ligands for the melanocortin receptors. Not surprisingly, many of these ligands are based upon recurring structural features found in melanocortin peptides. The Merck research group has reported a potent and receptor selective small molecule agonist for the MC4R.[96] This compound, based on the 4-substituted 4-cyclohexylpiperidine template (Fig. 13A), is the first literature disclosure of a highly potent non-peptide ligand selective for the MC4R. The compound was found to significantly inhibit food intake[63,96] and increased the erectile response[180] in rodent models, further validating the role of the MC4R in energy homeostasis and erectile activity. This compound does not contain a basic moiety that can mimic the Arg^8 side chain, although the compound is highly potent at the MC4R. These data further suggest that the spatial arrangement of the hydrophobic groups is an important factor in molecular recognition and activation of the melanocortin receptors. The significance of spatial arrangement of the hydrophobic residues may be inferred from a comparison of

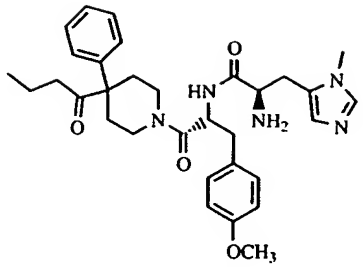
A



Receptor	EC_{50}(nM)
hMC1R	2850
hMC3R	2487
hMC4R	**2.1**
hMC5R	737

Ref. 96

B



Receptor	EC_{50}(nM)
hMC1R	**28**
hMC3R	NA
hMC4R	2600
hMC5R	4400

Ref. 51

Figure 13. Potent and selective small molecule agonists for the MC4R (**A**) and MC1R (**B**). Data from Refs.[96] (A) and[51] (B).

the low energy conformer of this small molecule with the low energy conformer of MTII. The orientation of the hydrophobic functionalities of this compound is very similar to the orientation of hydrophobic side chains in the low energy conformer of MTII.[96] The scientists at Bristol-Myers Squibb have reported the first highly potent and selective small molecule agonist for the MC1R.[51] This compound is based on a 4-substituted 4-phenylpiperidine template and has many structural features similar to the Merck MC4R selective small molecule (Fig. 13B). This compound was efficacious in an *in vivo* model for acute inflammation, demonstrating the role of the MC1R in inflammation.

In addition to the above small molecule ligands, other non-peptide compounds have been identified that interact with specific melanocortin receptors. Structures of various peptidomimetic ligands reported for the melanocortin receptors are shown in Figure 14. Our research group has recently synthesized a series of urea compounds (Fig. 14A) based on the Phe-Trp-Lys tripeptide sequence and screened them for agonist activity at the MC1R, MC3R–MC5R.[182] Many of the urea based ligands activated the melanocortin receptors with potencies ranging from micromolar to nanomolar. Kulesza et al.[183] have reported trisubstituted tetrahydropyrans (Fig. 14B) that bind to the MC4R with affinities similar to that of the DPhe-Arg-Trp-NH_2 tripeptide. Mutulis et al.[97,98] have used both *N*-alkyl amino acid derivatives and reductive amination products (Fig. 14C–E) to obtain non-peptide compounds that bind with micromolar affinities to the MC1R and MC3R–MC5R. Likewise to the reported small molecule agonists of the melanocortin receptors, non-peptide molecules have recently been disclosed in the literature that bind to the MC4R and antagonize the activity of α-MSH. Using two peptoid scaffolds, Millhauser et al. have designed compounds that mimic the core Arg-Phe-Phe sequence of AGRP. One of the peptoids was a functional antagonist at the MC4R (Fig. 14F), providing an important lead in the design of AGRP mimetics.[181] Scientists at Amgen have pursued the design of non-peptide ligands (Fig. 14G) with the ability to inhibit AGRP binding to the MC4R.[100] The goal of the Amgen study was to inhibit AGRP interactions with the MC4R without interfering with agonist activation of receptor, however, the compounds were determined to inhibit the activity of both AGRP and the endogenous agonist α-MSH.

The studies discussed above illustrate the progression from peptide ligands to that of non-peptide ligands for the melanocortin receptors. Many of the above compounds have improved properties, such

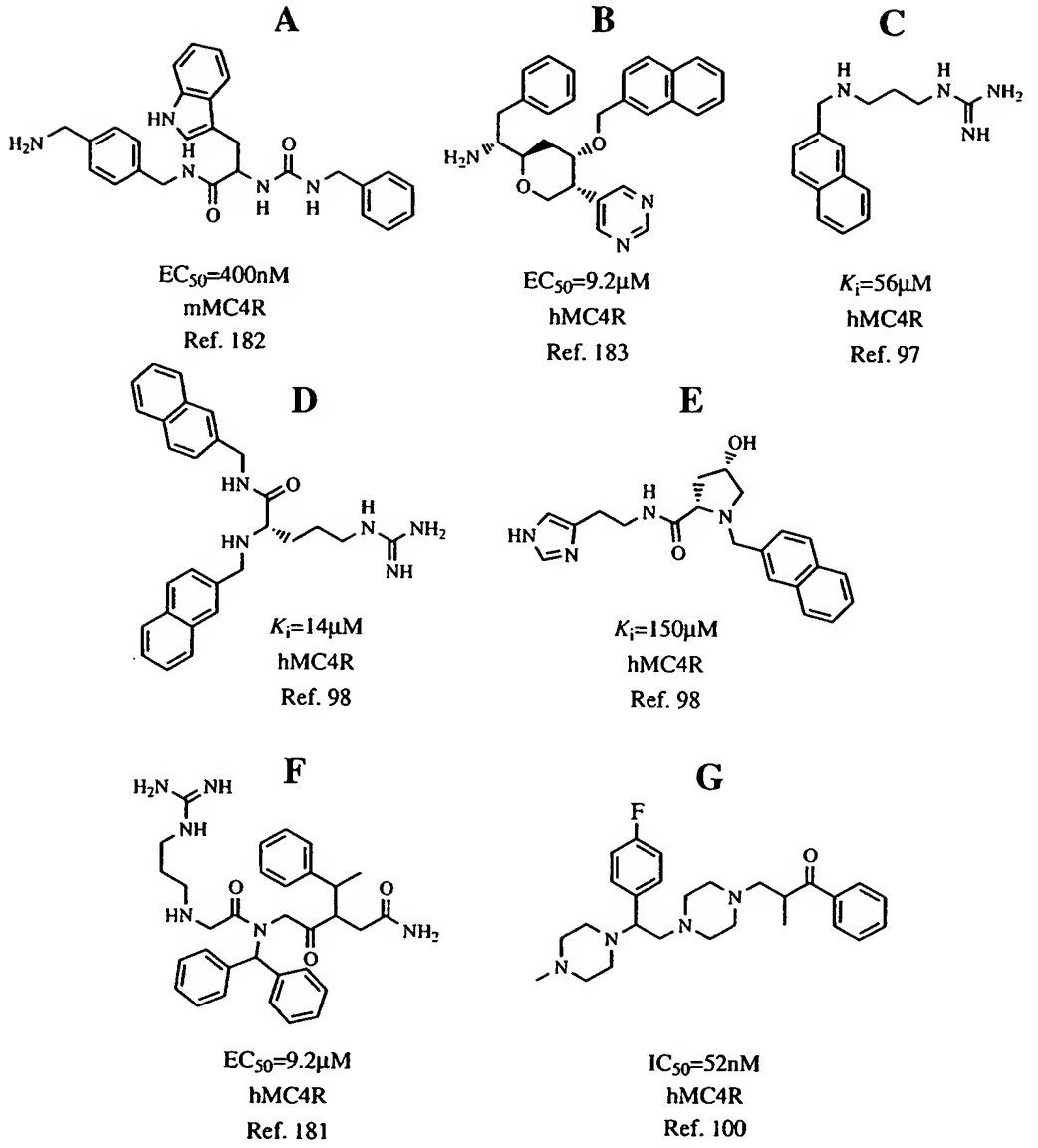


Figure 14. Various peptidomimetic compounds reported for the melanocortin receptors. Functional and binding activities shown are for the MC4R. Data from Refs.[97,98,100,181–183]

as potency, selectivity, and bioavailability, as compared with the properties of lead peptides. The non-peptide compounds have provided experimental evidence to support the hypothesis regarding the bioactive conformation of peptide ligands[94,95] and have linked specific melanocortin receptors with physiological functions.[51,96] The absence of basic functionalities in many of the non-peptide compounds suggest that the guanidine group found in the common "core" sequence of melanocortin peptides may not be essential to activity, if the hydrophobic moieties are in the correct spatial arrangement. The compounds above demonstrate that small molecule ligands for the melanocortin receptors are a viable option when considering melanocortin ligands for clinical applications. The recent advances in development of potent and selective non-peptide ligands surely will enhance understanding the exact physiological roles of this important receptor family.

9. CONCLUSIONS

Considerable effort has been made in an attempt to understand the interactions that occur in formation of stable ligand–melanocortin receptor complexes, and areas of peptidomimetic research continue to advance the design (rationale and random) of ligands that successfully mimic the biological activities of endogenous melanocortin peptides. Although much has been learned from the various SAR studies of melanocortin ligands, it still remains difficult to design peptidomimetic ligands *de novo* with specific activities at specific melanocortin receptors. The rationale design approach to peptidomimetic research is both challenging and fascinating, and new discoveries continue to emerge from these strategies involved in this approach. The results from SAR studies have enhanced our understanding of the melanocortin receptor system, and may expectantly aid in the design of novel ligands with optimized potency, stability, and receptor subtype selectivity.

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